Heparin-dependent binding and autophosphorylation of epidermal growth factor (EGF) receptor by heparin-binding EGF-like growth factor but not by EGF

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ABSTRACT Heparin-binding EGF-like growth factor (HB-EGF) is a recently identified member of the EGF family of growth factors and a potent mitogen for smooth muscle cells and fibroblasts. Chinese hamster ovary (CHO) cells genetically engineered to express the human EGF receptor bind with high affinity both EGF and HB-EGF. CHO mutant cells lacking heparan sulfate proteoglycans (HSPG) bind EGF equally well to wild-type cells and EGF binding is not affected by exogenous heparin. However, HSPG-deficient EGF receptor-expressing cells do not bind significant levels of HB-EGF unless heparin is present in the binding medium. Moreover, binding of radio-labeled EGF to HSPG-deficient EGF receptor-expressing cells is efficiently displaced by nonlabeled HB-EGF only in the presence of heparin. Signal transduction by the EGF receptor tyrosine kinase as evidenced by receptor autophosphorylation is induced by HB-EGF only in the presence of heparin, in contrast to EGF-induced receptor autophosphorylation, which is independent of heparin. These results directly demonstrate that HB-EGF but not EGF requires heparin or cell surface HSPG for binding and activation of the EGF receptor and that HB-EGF receptor interactions can be tightly regulated by the available local concentration of heparin-like molecules.

Heparin-binding EGF-like growth factor (HB-EGF) is a potent mitogen for smooth muscle cells and fibroblasts first identified as a secreted product of cultured human macrophages (1, 2) and is widespread in adult mammalian tissues (3) and wound fluids (4). It is a member of the EGF family, which includes EGF, type α transforming growth factor, amphiregulin, vaccinia growth factor, the neu differentiation factor (5), and the recently identified betacellulin (6). They all share high sequence homology including six conserved cysteine residues, which are similarly spaced in the C-terminal part of the molecule (7). HB-EGF is initially synthesized as a large precursor of 208 amino acids, which is eventually cleaved to yield the mature protein of at least 86 amino acids and a number of variants with N-terminal microheterogeneity. HB-EGF, as well as amphiregulin and neu differentiation factor has a 30- to 35-amino acid extension upstream from the EGF-like domain that is extremely hydrophilic due to the abundance of lysine and arginine residues (7). This cationic N-terminal extension is most probably responsible for the relatively high affinity of these growth factors for the glycosaminoglycan heparin.

A variety of growth factors have been characterized that form tight complexes with heparin and heparan sulfates (HS) including members of the fibroblast growth factor (FGF) family (8), vascular endothelial growth factor (9), HB-EGF (2), and the cytokines interleukin 3, granulocyte–macrophage colony-stimulating factor (10), and interferon γ (11). Basic FGF (bFGF) was identified as a complex with HS proteoglycans (HSPG) on cell surfaces and basement membranes of diverse tissues and blood vessels (12, 13). HSPG-bound bFGF is protected against heat inactivation and proteolytic degradation (14). Recent studies identified an unusual role for heparin-like molecules in the formation of distinct bFGF–heparin complexes that are essential for binding of bFGF to its cognate receptor (15). The crucial role of cell surface HS was revealed by the finding that high-affinity receptor binding of bFGF was abolished in Chinese hamster ovary (CHO) mutant cell lines defective in their metabolism of glycosaminoglycans and receptor binding was restored upon addition of exogenous heparin (15). Several studies have since shown that members of the FGF family require heparin for binding to their receptors and for biological activity (16–20), and this interaction requires specific heparin structures (21–23). As both EGF and HB-EGF seem to share the same high-affinity receptors (7), it is of great interest to determine the differential effects of heparin on the interactions of these growth factors with their receptor. To address this question, we have investigated the binding and activation of the EGF receptor by EGF and HB-EGF in wild-type and heparan sulfate-deficient CHO mutant cells genetically engineered to express the human EGF receptor.

EXPERIMENTAL PROCEDURES

Cell Lines Expressing the Human EGF Receptor. CHO wild-type cells and CHO-745 mutant cells defective in their metabolism of glycosaminoglycans (24) were cotransfected with the plasmids pLSV encoding the EGF receptor (25) and pSV2-Neo encoding the neomycin resistance gene by the calcium phosphate method. Transfected cells were selected and maintained in F-12 medium supplemented with 10% fetal calf serum (HyClone), 1% glutamine, and neomycin (0.5 mg/ml) (GIBCO). Individual clones expressing the EGF receptor were identified by Western blot analysis with an anti-EGF receptor antibody (26) and by their ability to bind 125I-labeled EGF (23-1-EGF class). No significant binding of EGF is observed on nontransfected, wild-type, or HSPG-deficient CHO cells.

Iodination of HB-EGF and EGF. Purified human HB-EGF (a generous gift from Michael Klagsbrun, Children's Hospital, Harvard Medical School, Boston) or recombinant HB-EGF (kindly provided by Judith Abrahams, Scios Nova) and EGF (Pepro-Tech, Boston) were labeled with 125I (American) by using the chloramine-T method (27). The HB-EGF reaction mixture was loaded on a 0.2-ml heparin Sepharose column (Pharmacia) and eluted from the column with 1 ml of elution buffer (20 mM sodium phosphate buffer, pH 7.2/2 M NaCl/0.1% CHAPS [3{[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate}). 123I-EGF was separated from free

Abbreviations: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan(s); FGF, fibroblast growth factor; bFGF, basic FGF.
125I on a Sephadex G-25 column. Specific activity of the ligands was 1.2 x 10^6 cpm/ng for purified HB-EGF, 1.1 x 10^5 cpm/ng for EGF, and 2.2 x 10^5 cpm/ng for human recombinant HB-EGF.

**Binding of 125I-EGF and 125I-HB-EGF to Cells.** Confluent cultures of cells in 24-well plates (Falcon) were precooled and washed twice with cold Dulbecco's modified Eagle's medium (DME) supplemented with 20 mM Hepes (pH 7.5) and 0.1% bovine serum albumin (DME/BSA). Subsequently, the cells were incubated for 1.5 h at 4°C with 125I-EGF or 125I-HB-EGF (2 ng/ml) in DME/BSA and different concentrations of heparin (Heparin, Franklin, OH) or with heparin (50 ng/ml) and increasing concentrations of unlabeled ligands. The binding medium was discarded and the cells were washed twice with ice-cold DME/BSA. To determine receptor binding of 125I-EGF or 125I-HB-EGF, the cells were incubated for 5 min with 0.5 ml of 1.6 M NaCl/10 mM sodium acetate, pH 4, which was then removed and assayed in a γ counter. Nonspecific binding was determined as the value obtained for receptor binding in the presence of a 100-fold excess of nonlabeled EGF or HB-EGF.

**Covalent Cross-Linking of Radiolabeled EGF and HB-EGF to EGF Receptor-Expressing Cells.** Confluent monolayers of EGF receptor-expressing cells were grown in 65-mm diameter plates. Binding of 125I-EGF or 125I-HB-EGF to the cells was carried out as described above. After 90 min, disuccinimidyl suberate (Pierce) in phosphate-buffered saline (PBS) was added to a final concentration of 0.15 mM and incubated for 30 min at room temperature. The cells were washed with PBS and scraped into 1 ml of lysis buffer [20 mM Hepes, pH 7.5/150 mM NaCl/10% glycerol/1% Triton X-100/1.5 mM MgCl2/1 mM EGTA/1 mM sodium orthovanadate/1 mM phenylmethylsulfonyl fluoride/aprotinin (1 μg/ml)/leupeptin (1 μg/ml)] and incubated for 10 min on ice. The cell lysates were cleared by centrifugation (12,000 × g; 15 min at 4°C) and boiled for 5 min in sample buffer [50 mM Tris-HCl, pH 6.8/25% (wt/vol) glycerol/6% 2-mercaptoethanol/4% SDS/1 mM EDTA/10% bromophenol blue], and loaded onto an SDS/7.5% polyacrylamide gel. After electrophoresis the gel was dried and exposed to x-ray film for 36 h.

**EGF Receptor Immunoprecipitation and Autophosphorylation Analysis.** Confluent HSPG-deficient, EGF receptor-expressing cells were grown in 65-mm diameter plates, starved in 0.1% serum for 12 h, and then stimulated by addition of EGF or HB-EGF in DME in 5 ng/ml for 10 min at 37°C. Cell lysates were incubated for 90 min at 4°C with protein A-agarose beads coupled to an anti-EGF receptor antibody (26). The immunoprecipitates were washed three times with HNTG (20 mM Hepes, pH 7.5/150 mM NaCl/10% glycerol/1% Triton X-100), separated on an SDS/7.5% polyacrylamide gel, and transferred onto nitrocellulose. The blot was incubated with antibodies to phosphotyrosine for 1 h at 4°C in Tris-buffered saline (10 mM Tris-HCl, pH 7.4/150 mM NaCl) containing 10% low-fat milk and 1% ovalbumin, reacted for 45 min at room temperature with horseradish peroxidase-conjugated protein A, and visualized by fluorography (ECL, Amersham).

**RESULTS**

**CHO Mutant Cells Transfected with the Human EGF Receptor as a Model System for Studying Heparin-Dependent Growth Factor–Receptor Interactions.** Stable clones of wild-type CHO and HS-deficient mutant cells pgsA CHO-745 (24) were obtained after transfection and selection for expression of the human EGF receptor. Clones of wild-type CHO and mutant cell pgsA CHO-745 cells were selected by Western blot analysis with anti-EGF receptor antibodies (26) and by their capacity to bind radiolabeled EGF to express comparable amounts of receptor. Cells derived from a single selected clone expressing moderately high levels of EGF receptors were tested for their ability to bind EGF and HB-EGF in the absence and presence of heparin. EGF receptor-expressing wild-type CHO cells demonstrated high levels of HB-EGF binding in the absence or presence of exogenous heparin with no potentiating effect of heparin on the binding of HB-EGF at concentrations as high as 5 μg/ml (Fig. 1A). In contrast, HS-deficient cells expressing the EGF receptor show very little HB-EGF binding in the absence of heparin (Fig. 1A). Binding of HB-EGF to these mutant cells was dramatically enhanced and fully restored when heparin was included in the binding medium. Heparin induces HB-EGF binding in a dose-dependent manner; furthermore, the binding curve plateaus, indicating saturation of the available HB-EGF binding sites (Fig. 1A). Maximal receptor binding is achieved with heparin concentrations around 0.5 μg/ml for purified HB-EGF and 0.1-0.2 μg/ml for recombinant HB-EGF (data not shown). 125I-EGF, on the other hand, bound equally well to wild-type and HS-deficient cells expressing the EGF receptor with or without exogenous heparin (Fig. 1B).

To unequivocally demonstrate that EGF and HB-EGF interact and with similar affinity with the EGF receptor and that heparin-dependent binding of HB-EGF did not result from any modification of the ligand during iodination (28), we tested the capacity of nonlabeled, recombinant EGF and HB-EGF to displace 125I-EGF on HSPG-deficient cells in the absence and presence of heparin (Fig. 2). Unlabeled EGF completely inhibited 125I-EGF binding at a concentration of

![Figure 1](image-url)  
**Fig. 1.** Effect of heparin on the binding of 125I-EGF and 125I-HB-EGF to wild-type and HS-deficient CHO cells expressing EGF receptor. Purified human HB-EGF (A) or EGF (B) (2 ng/ml) was incubated with wild-type CHO-K1 cells or HS-deficient CHO-745 mutant cells, both expressing EGF receptor type 1, for 90 min at 4°C with increasing concentrations of heparin. Results represent one of three independent experiments. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled ligand and did not exceed 20% of the total bound ligand.
100 ng/ml and with a calculated apparent dissociation constant of 2–4 nM with or without heparin (Fig. 2A). HB-EGF, on the other hand, could not efficiently compete on the binding of 125I-EGF unless heparin was included in the binding medium (Fig. 2B). In the presence of heparin (50 ng/ml), HB-EGF at 100 ng/ml completely blocked 125I-EGF binding and with an apparent dissociation constant of 1 nM. Taken together, these results strongly suggest that heparin or cell surface HSPG are required for binding of HB-EGF but not of EGF to the EGF receptor.

**Chemical Cross-Linking of 125I-EGF or 125I-HB-EGF to Wild-Type and HS-Deficient CHO Cells and Its Modulation by Heparin.** Chemical cross-linking of 125I-EGF and 125I-HB-EGF to wild-type and HS-deficient cells further demonstrates that binding of HB-EGF requires heparin. We demonstrate the formation of a labeled ligand–receptor protein complex with an expected apparent molecular mass of 185 kDa on wild-type as well as on the HS-deficient cells (Fig. 3). Cross-linking of 125I-HB-EGF to wild-type cells results in an HB-EGF–receptor protein complex of an apparent molecular mass greater than 200 kDa. In contrast, no detectable labeled protein complex was obtained by cross-linking 125I-HB-EGF to HS-deficient EGF receptor-expressing cells, unless heparin was included in the binding medium prior to cross-linking (Fig. 3). These results are in complete agreement with the radioreceptor binding data shown in Fig. 1A and provide further evidence that HB-EGF cannot interact with the EGF receptor on HS-deficient cells and that heparin or cell surface HSPG can interchangeably promote high-affinity receptor binding of HB-EGF.

**Heparin-Dependent Activation of the EGF Receptor Tyrosine Kinase.** To test the effects of heparin on receptor activation and signal transduction by HB-EGF, we analyzed ligand-induced tyrosine kinase activity of the EGF receptors expressed in HS-deficient cells in the absence and presence of heparin. Phosphorylated EGF receptor was immunosolated using an anti-EGF receptor-specific antibody and visualized by immunoblotting with antibodies directed against phosphotyrosine. As shown in Fig. 4, EGF receptor autophosphorylation is stimulated by EGF equally well in wild-type and HS-deficient cells with no additional effects of exogenous heparin. In contrast, without heparin HB-EGF does not stimulate the EGF receptor tyrosine kinase above basal phosphorylation level of the receptor detected in the absence of any added ligand. The addition of heparin, however, fully reconstitutes HB-EGF-induced EGF receptor autophosphorylation and tyrosine kinase activity in EGF receptor-expressing HSPG-deficient cells (Fig. 4).

**DISCUSSION**

There is increasing evidence to support the hypothesis that binding of some GFGs to their high-affinity receptors is regulated by cell surface HSPG (15–18). Here, our results suggest that HB-EGF, which is apparently unrelated to the FGF family, also requires cell surface HSPG in order to bind and activate its high-affinity receptor. We present evidence that wild-type CHO cells transfected with the EGF receptor efficiently bind HB-EGF, whereas mutant HS-deficient CHO cells do not. Moreover, HB-EGF binding as well as its activation are highly regulated by cell surface HSPG.

**Fig. 4.** Ligand-induced heparin-dependent tyrosine phosphorylation of EGF receptors. Western blot analysis of tyrosine phosphorylated proteins in wild-type and HS-deficient CHO cells stimulated with EGF or HB-EGF. EGF receptor-expressing CHO-745 cells were stimulated with EGF or HB-EGF (5 ng/ml) in DMEM/BSA at 37°C in the absence and presence of heparin (1 µg/ml). Receptor immunoprecipitates were separated on an SDS/7.5% polyacrylamide gel, blotted onto nitrocellulose, and reacted with rabbit antibodies directed to phosphotyrosine.
capacity to displace EGF from HSPG-deficient cells can be fully restored in a dose-dependent manner by including heparin in the binding medium. This is in marked contrast to the binding of EGF to the same receptor, which appears to be absolutely heparin independent. Moreover, signal transduction by the EGF receptor, as evidenced by receptor auto-phosphorylation, is stimulated by HB-EGF only in the presence of heparin or intact cell surface HSPG. These results directly demonstrate that HB-EGF but not EGF requires heparin or cell surface HSPG for binding and activating its high-affinity receptor. This may also help explain the recent observations that heparin potentiates HB-EGF-induced migration of smooth muscle cells (29) and mitogenesis in mouse epidermal keratinocytes (4) and is consistent with the findings that heparin-binding peptides derived from HB-EGF as well as heparinase pretreatment of cells modulate HB-EGF binding and biological activity (29, 30).

Several models have been proposed to help explain heparin-dependent growth factor–receptor interactions. In the original model, it was suggested that interaction of bFGF with cell surface HSPG leads to a conformational change in the growth factor enabling the formation of an active bFGF, heparin, and FGF receptor trimolecular complex (15). This induced fit model has recently been supported by direct physical measurements of infrared spectroscopy demonstrating an induced conformational change in bFGF after binding to heparin (31). Our present study further contribute to the understanding of heparin-dependent growth factor–receptor interaction as it provides a demonstration of heparin-dependent and independent binding of two growth factors to one receptor.

It has been proposed that heparin-like molecules may induce the formation of active FGF dimers leading to FGF receptor dimerization and trans-activation (32). Unlike FGF receptors, it is well established that dimerization of EGF receptors is an intrinsic property of the receptor molecules, which, although triggered by ligand binding, involves direct physical interaction of the two monomeric receptors (33, 34). Moreover, oncogenic receptors in the EGF receptor family like the HER2/neu tyrosine kinase form homo- and heterodimers independent of ligand binding (35). As EGF and HB-EGF differ mainly in their N-terminal region, which is responsible for heparin binding, it is somewhat unlikely that EGF receptor dimerization involves different molecular mechanisms for the two ligands. This implies that HB-EGF-induced receptor dimerization is most probably not a function of heparin-mediated ligand dimerization.

In a recent study, Kan and colleagues (36) have demonstrated that heparin can interact independently of the FGF ligand with a specific sequence in the extracellular domain of the FGF receptor and that this interaction is essential for binding of FGF to the receptor. Both EGF and HB-EGF share a common receptor that binds EGF regardless of cell surface HSPG but fails to bind HB-EGF unless HSPG or heparin is present. This effect of heparin on HB-EGF but not on EGF binding suggests that heparin most likely regulates ligand–EGF receptor interaction via binding to the ligand and not to the receptor. Nevertheless, one cannot exclude the possibility that cell surface HSPG could selectively determine the receptor’s capacity to bind heparin-binding ligands such as HB-EGF, amphiregulin, and neu differentiation factor and not be involved in the binding of non-heparin-binding ligands such as EGF and type α transforming growth factor. It is, therefore, of great interest to determine whether EGF receptors possess an intrinsic heparin-binding site in their extracellular domain similar to that proposed for FGF receptors (36).

While binding of HB-EGF to the EGF receptor is enhanced at relatively low levels of heparin, higher heparin concentrations lead to inhibition of receptor binding similar to that observed with FGF. In a recent study analyzing the capacity of HB-EGF to compete for binding of $^{125}$I-EGF to A-431 cells, apart from the stimulatory effects of heparin, the major effect followed was a potent inhibitory activity of heparin on the capacity of HB-EGF to compete on $^{125}$I-EGF receptor binding (30). The molecular mechanism underlying this biphasic effect of heparin on heparin-binding growth factors is not known. However, we have recently demonstrated that several cell surface-derived HSPG can inhibit bFGF receptor binding by direct competition with heparin (22), and several recent reports have identified specific heparin structures that bind bFGF with various affinities (37, 38). We hypothesized that both stimulatory and inhibitory types of HS exist that either promote or restrict bFGF receptor binding (22). The balance between these two classes of HS may well contribute to the biphasic effects observed as well as determine the degree and extent of HB-EGF-induced cellular responses.

Ligand binding to receptor tyrosine kinases is followed by receptor dimerization, stimulation of protein tyrosine kinase activity, and autophosphorylation (33). Here we show that not only HB-EGF binding but all subsequent steps in signal transduction leading to receptor autophosphorylation are heparin dependent. The lack of any enhancement of EGF receptor autophosphorylation by HB-EGF above background in the absence of exogenous heparin strengthens the notion that heparin-like molecules may be an absolute requirement for HB-EGF receptor interaction. Heparin-dependent activation and attenuation of HB-EGF receptor interactions may be expected to have marked physiological implications. For example, the action of HB-EGF secreted by macrophages in wound healing and arteriosclerotic lesions could be dramatically potentiated by local cellular- and tissue-specific HSPG. The differential capacity of heparin and HS to promote receptor binding of HB-EGF but not EGF could have spatial functional significance in determining the more diffuse, widespread action of EGF on the one hand and a highly restricted local activity of HB-EGF on the other. As such, the local concentration and activation of HB-EGF but not EGF by HSPG may well explain the observation that HB-EGF is by far a more potent mitogen and migration factor for vascular smooth muscle cells than EGF (29).
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